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Identification of Downstream-Initiated c-Myc Proteins Which Are Dominant-Negative Inhibitors of Transactivation by Full-Length c-Myc Proteins

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The c-*myc* gene has been implicated in multiple cellular processes including proliferation, differentiation, and apoptosis. In addition to the full-length c-Myc 1 and 2 proteins, we have found that human, murine, and avian cells express smaller c-Myc proteins arising from translational initiation at conserved downstream AUG codons. These c-Myc short (c-Myc S) proteins lack most of the N-terminal transactivation domain but retain the C-terminal protein dimerization and DNA binding domains. As with full-length c-Myc proteins, the c-Myc S proteins appear to be localized to the nucleus, are relatively unstable, and are phosphorylated. Significant levels of c-Myc S, often approaching the levels of full-length c-Myc, are transiently observed during the rapid growth phase of several different types of cells. Optimization of the upstream initiation codons resulted in greatly reduced synthesis of the c-Myc S proteins, suggesting that a "leaky scanning" mechanism leads to the translation of these proteins. In some hematopoietic tumor cell lines having altered c-*myc* genes, the c-Myc S proteins are constitutively expressed at levels equivalent to that of full-length c-Myc. As predicted, the c-Myc S proteins are unable to activate transcription and inhibited transactivation by full-length c-Myc proteins, suggesting a dominant-negative inhibitory function. While these transcriptional inhibitors would not be expected to function as full-length c-Myc, the occurrence of tumors which express constitutive high levels of c-Myc S and their transient synthesis during rapid cell growth suggest that these proteins do not interfere with the growth-promoting functions of full-length c-Myc.

Increasing evidence suggests that members of the *myc* family of proto-oncogenes function as regulators of gene transcription and perform a fundamental role in the control of cell growth and differentiation (25, 36, 46). This is dramatically illustrated by the frequent occurrence of a variety of tumors in many species having alterations of *myc* genes and the transduction of c-*myc* sequences by retroviruses (48). The diverse biological activity of *myc* is demonstrated by its ability to contribute to cellular proliferation (48), inhibit terminal differentiation (9), and promote apoptosis (14). However, despite intensive study, the mechanism by which Myc proteins perform such diverse cellular roles is unknown (25, 36, 46).

Myc proteins have many features in common with other transcriptional regulators. The major c-Myc proteins are phosphorylated, localized to the nucleus, and have relatively short half-lives (21, 45). Specific molecular functions have been assigned to both the C-terminal and the N-terminal regions of the proteins. The C-terminal domain of the Myc proteins is structurally similar to a superfamily of transcription factors having a cluster of basic amino acids necessary for sequence-specific DNA binding adjacent to a basic helix-loop-helix–leucine zipper motif (bHLH-LZ) (36). Dimerization of Myc with Max, another bHLH-LZ protein (7, 43), through the HLH-LZ region of the two proteins, allows sequence-specific binding to CACGTG or E-box Myc site (EMS) DNA sequence (6, 44). The c-Myc protein can stimulate transcription through EMS sequences, while excess Max protein antagonizes this transactivation in mammalian and yeast cells (1, 2, 32).

Transcriptional activation by the c-Myc protein is also dependent on an intact N-terminal domain as well as the C-terminal domain. The N-terminal region of the c-Myc protein functions as a transactivation domain when fused to the DNA binding portion of the Gal4 protein (26), and deletions of the highly conserved areas within the N-terminal domain (*myc* boxes) reduce its transactivation ability (26, 32). In addition, transactivation may be modulated by proteins interacting with the N-terminal domain. The TATA-binding protein (TBP) and the pRb-related protein, p107, have both been shown to interact with the N-terminal domain of c-Myc (18, 39).

An unusual feature of a growing number of transcription factors and oncogenes including *myc* is the ability to synthesize alternative translational forms from multiple initiator codons. Two major c-Myc proteins, c-Myc 1 and 2, have been found in all vertebrate species examined thus far (22). In mammalian and avian cells, the two proteins arise from alternative initiation at in-frame non-AUG and AUG codons (22). Alterations of the c-*myc* locus can result in disrupted synthesis of the two alternatively initiated proteins. In Burkitt's lymphoma cells, rearrangement of the c-*myc* locus by chromosomal translocation or specific point mutations commonly results in the loss of c-Myc 1 protein expression (21, 22). In addition, in cells having intact c-*myc* genes, the alternative translational initiation of c-Myc can be regulated during cell growth. The c-Myc 2 protein is the predominant form expressed in growing cells, while synthesis of the upstream, non-AUG-initiated c-Myc 1 protein increases dramatically to levels equal to or greater than those of c-Myc 2 as the cells approach high-density growth arrest (23). There also appear to be functional differences between the c-Myc 1 and 2 proteins. The c-Myc 1 protein, in contrast to c-Myc 2, can transactivate through a noncanonical site, the

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C/EBP binding sequences found in the EFII viral enhancer (20). Also, overexpression of c-Myc 1, unlike that of c-Myc 2, inhibits the growth of COS cells (20).

In this report, we demonstrate that human, murine, and avian cells express smaller c-Myc proteins in addition to the full-length c-Myc 1 and 2 proteins. These smaller proteins, termed c-Myc S, arise from translational initiation at conserved AUG codons downstream of the initiation sites for c-Myc 1 and 2. The c-Myc S proteins have a severely truncated N-terminal transactivation domain but retain the C-terminal protein dimerization and DNA binding domain. Similarly to full-length c-Myc proteins, the c-Myc S proteins appear to be localized to the nucleus, are relatively unstable, and are phosphorylated. In contrast to the full-length Myc proteins, the synthesis of the c-Myc S proteins is transient during growth. An exception to this transient expression is found in some hematopoietic tumor cell lines having altered c-*myc* genes which synthesize the c-Myc S proteins at constitutive levels equivalent to that of full-length c-Myc 2 protein. The expression of the c-Myc S proteins would be expected to have an inhibitory or dominant-negative effect on the function(s) of the full-length c-Myc proteins. Indeed, we found that the c-Myc S proteins, which lack a conserved region in the N-terminal transactivation domain, inhibit transactivation by full-length c-Myc proteins. However, the occurrence of tumors which express constitutive high levels of c-Myc S and their transient synthesis during rapid cell growth suggest that these proteins do not interfere with the growth-promoting functions of full-length c-Myc.

MATERIALS AND METHODS

Cell lines. The APRT$^-$ subclone of murine erythroleukemia (MEL) cell line 745 was obtained from D. Miller (Fred Hutchinson Cancer Research Center, Seattle, Wash.). The murine fibroblast cell line NIH 3T3 was obtained from the American Type Culture Collection (ATCC), Bethesda, Md. COS-7 cells were obtained from S. Brandt (Vanderbilt University, Nashville, Tenn.). The avian bursal lymphoma cell lines Bk3A, Bk25, H1, S13, 243L1, 249L4, and 293Sc and the Mark's virus-infected cell line MSB-1 were obtained from Maxine Linial (Fred Hutchinson Cancer Research Center). The human colon carcinoma cell line COLO-320 was obtained from M. Groudine (Fred Hutchinson Cancer Research Center). The COS-7 cells were maintained in Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) supplemented with 10% defined-supplemented calf serum (CS) (HyClone Laboratories, Logan, Utah). The avian bursal cell lines and MSB-1 cells were cultured in suspension cell medium (SCM) consisting of DMEM supplemented with 10% tryptose phosphate broth (Gibco Laboratories, Grand Island, N.Y.), 5% CS, and 1% heat-inactivated chick serum (Gibco). The MEL cell lines were cultured in DMEM-HG (Gibco) supplemented with 5% fetal CS (HyClone) and 5% CS (defined-supplemented; HyClone), along with 500 U of penicillin-streptomycin (1:1; Gibco) per ml, and were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Plasmids and site-directed mutagenesis. The 1.5-kb *Sac*I-*Hin*dIII fragment of a wild-type murine c-*myc* cDNA (MM) containing the entire protein coding frame and the pKSmax1.8 plasmid containing a 1.8-kb cDNA with the entire coding frame for murine Max (p21) were obtained from Michael Cole (Princeton University, Princeton, N.J.). Plasmid pSLCM containing the 1.8-kb *Eco*RI fragment of the chicken c-*myc* cDNA (41) with the entire coding frame was obtained from Maxine Linial (Fred Hutchinson Cancer Research Center).

To enable our avian c-Myc-specific antiserum (anti-av-Myc12C) to selectively immunoprecipitate the exogenous murine c-Myc proteins and not the endogenous murine or human c-Myc proteins, the penultimate C-terminal glycine of the MM cDNA was previously changed to an arginine to yield the MM(c) cDNA (20). The MM(c) cDNA was inserted into the *Sac*I-*Hin*dIII sites of plasmid pGEM 1 (Promega). The 1.5-kb *Eco*RI-*Hin*dIII fragments from these shuttle plasmids were then inserted into the same sites of pBluescript II SK(−) (Stratagene). The cDNA inserts were oriented in such a way that in vitro transcription initiated at the T3 promoter produced sense RNA. The resulting plasmid (pBMM) was used for in vitro transcriptions and translations (see below). Plasmid pBMM(c) was utilized for deletion or site-directed mutagenesis as described below. The various murine c-*myc* mutant cDNAs derived from the pBMM(c) plasmid, except the deletion mutant pBMMΔ5′, were inserted into the *Not*I-*Apa*I polycloning sites of the eukaryotic expression vector pRc/CMV (InVitrogen). The pBMMΔ5′ and Max1.8 cDNAs were inserted into the *Not*I-*Xho*I sites of the eukaryotic expression vector pCEP4 (InVitrogen).

The chicken c-*myc* cDNA (CMfl) was inserted into the in vitro expression vector, Bluescript II SK(−). The chicken c-*myc* cDNA was truncated at the 5′ end by digestion of the pBCMfl plasmid with Pst-1 and religation of the linear plasmid, minus the Pst-1 fragment, upon itself creating pBCMΔ5′. For expression in vivo, the CMfl cDNA was inserted into the *Eco*RI sites of pGEM7zf+ in the SP6 sense orientation and then directly subcloned into the *Hin*dIII-*Xba*I sites of pRc/CMV. For expression of CMΔ5′ cDNA in vivo, the cDNA was first subcloned into the pGEM7zf+ *Sma*I-*Eco*RI polycloning site and then subcloned from this shuttle vector into the *Hin*dIII-*Xba*I sites of pRc/CMV. The human c-Myc 1 and 2 cDNAs in the retrovirus expression vector LXSN (LXSN-HM1 and LXSN-HM2) were obtained from R. Eisenman (Fred Hutchinson Cancer Research Center).

Plasmid pBMM(c) was used for isolation of single-stranded DNA template in the in vitro DNA synthesis reactions. Mutant oligonucleotides with the substituted base(s) located in the middle were purchased from Biosynthesis Inc. (Denton, Tex.) and used to prime DNA synthesis. Mutagenesis was carried out with the oligonucleotide-directed in vitro mutagenesis system, version 2 (Amersham Corporation, Amersham, England), under the conditions specified by the manufacturer. To confirm successful incorporation of altered nucleotides and the absence of spurious changes, mutant cDNAs were sequenced with a Sequenase 2 kit (U.S. Biochemical, Cleveland, Ohio).

Cell transfections. For transfection of COS cells, the cells were plated at a density of 2.0×10^6 to 2.5×10^6 cells per 100-mm dish (approximately 75% confluent for transient transfection) or at a density of 1.5×10^6 cells per 100-mm culture dish (approximately 50% confluent for stable transfections) and were allowed to recover overnight before transfection. COS cells were transfected by the calcium phosphate method (17). Briefly, the various cesium chloride-purified plasmids (10 to 20 μg for stable transfections or as described in the figure legends for transient transfections) were precipitated with calcium phosphate for 30 min and applied to the cells. The cells were then forced to take up the DNA by glycerol treatment 4 to 6 h later. After the cells were allowed to recover and grow for 48 h, the cells were harvested for analysis, or G418 (Gibco-BRL) was added to a final active concentration of 400 μg/ml. Individual G418-resistant colonies were removed from the culture dishes and cultured separately in the continuous presence of 400 μg of G418 per ml for 3 or more weeks. Individual clones were screened for exogenous c-Myc protein expression by immunoprecipitation analysis as described below.

Generation of antibodies. Affinity-purified chicken-, mouse-, and human-specific c-Myc peptide antibodies (anti-av-Myc12C, anti-mu-Myc12C, and anti-hu-Myc12C, respectively) were generated as previously described (19, 21, 49). Briefly, New Zealand White rabbits were inoculated with complete Freund's adjuvant containing 500 μg of either mouse, chicken, or human c-Myc C-terminal peptide (12-mer) conjugated to bovine serum albumin (BSA). The antisera were affinity purified by sequential chromatography on protein A-Sepharose, bovine serum albumin-conjugated Sepharose (to remove antibodies to BSA), and, finally, c-Myc peptide-conjugated Sepharose. The Max antiserum (anti-Max) was generated as described previously (20) and affinity purified by chromatography on His-Max-conjugated Sepharose. Anti-MycN100 and anti-Mycfl were generated by inoculating a New Zealand White rabbit with complete Freund's adjuvant containing 500 μg of either purified bacterially expressed murine full-length c-Myc fused to glutathione *S*-transferase (GST-Mycfl) or a purified bacterially expressed fragment of murine c-Myc containing the N-terminal 100 amino acids fused to GST (GST-MycN100). Both antisera were purified as described for anti-Max above.

Immunoprecipitation. Cell samples were collected (10^7 cells), washed twice with phosphate-buffered saline (PBS; pH 7.3), and labeled with 300 μCi of [^{35}S]methionine or [^{35}S]methionine and [^{35}S] cysteine together (^{35}S-Trans Label; ICN) in 1 ml of methionine-free medium (Gibco) for 20 min at 37°C. The cells were then washed twice with PBS and stored at −80°C until preparation for immunoprecipitation. Radiolabeled cells were solubilized in cold antibody (Ab) buffer (19) containing 10 mM iodoacetamide and were disrupted by sonication at 4°C. The amounts of radiolabel incorporated into cellular proteins were determined by precipitation onto filters (in duplicate) with 10% trichloroacetic acid (TCA). Equivalent amounts of TCA-precipitable counts from each cellular lysate were adjusted to equal volumes with Ab buffer and then precleared by incubation with *Staphylococcus aureus* membranes (Immuno-Precipitin; Bethesda Research Laboratories [BRL], Bethesda, Md.) and clarified by centrifugation (10 min at $10{,}000 \times g$) before addition of antibody. The c-Myc proteins were precipitated by the addition of 5 μg of appropriate antibodies. Immune complexes were precipitated with Immuno-Precipitin for 30 min. The immunoprecipitation complexes were washed three times with radioimmunoprecipitation assay (RIPA) buffer (19) and incubated at 95°C for 3 min in sample buffer. The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE [10% acrylamide]) and fluorographed as described by Skinner and Griswold (47). High-range protein molecular mass markers (BRL) were used as standards in each SDS-PAGE gel and were visualized by staining with Coomassie brilliant blue. Molecular mass standards consisted of myosin (200,000 Da), phosphorylase *b* (97,400 Da), BSA (68,400 Da), ovalbumin (43,000 Da), carbonic anhydrase (29,000 Da), β-lactoglobulin (18,400 Da), and lysozyme (14,300 Da).

Cellular fractionation analysis. Avian 243L1 cells were metabolically labeled with [^{35}S]methionine and [^{35}S] cysteine for 30 min, washed twice with Dulbecco's PBS (DPBS; pH 7.3), and placed on ice. A third of the sample was lysed in complete Ab buffer to yield whole-cell lysate (WCL). The rest of the cells were

resuspended in either TNT (20 mM Tris [pH 7.4], 50 mM NaCl, 0.5% Triton X-100) or TTN (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Triton X-100), and the suspensions were incubated on ice for 10 min. The insoluble components were separated from the soluble components by centrifugation (400 $\times$ *g*) for 5 min at 4°C. The supernatants were transferred to another tube, and the remaining insoluble fractions were washed once with the TNT or TTN buffer to remove contaminating supernatant and were resuspended in the appropriate extraction buffer. The soluble and insoluble fractions were then adjusted with SDS and deoxycholate to the same final concentrations found in Ab buffer. The c-Myc proteins were immunoprecipitated from equal volumes of each lysate by using the anti-av-Myc12C antibody as described above.

Protein turnover measurement. Cells (2.5 $\times$ 10^7) were collected at various times after treatment as indicated in the figure legends, washed twice with PBS, and labeled with 500 μCi of [^{35}S]methionine for 10 min (pulse) as described above. The cells were then washed with PBS (pH 7.3), resuspended in warm growth medium, and incubated at 37°C. Cell samples (5 $\times$ 10^6) were collected at the specified time intervals (chase), washed twice with PBS, and stored at −80°C until preparation for immunoprecipitation as described above.

In vitro transcription and translation. For in vitro transcription, the various plasmids were linearized at the 3′ end by digestion with restriction enzymes and purified by phenol-chloroform extraction, followed by ethanol precipitation. In vitro transcription of the purified, linearized plasmids was performed with the MCAP kit (Stratagene) exactly as specified by the manufacturer. The transcribed RNAs were purified by RQ1 (RNase-free) DNase I digestion (Promega) and phenol-chloroform extraction, followed by ethanol precipitation. Purified RNAs (500 ng to 1 μg) were translated in vitro with rabbit reticulocyte lysate systems (Promega and BRL) in the presence of 50 μCi of [^{35}S]methionine. The proteins were immunoprecipitated as described or were immediately separated by SDS-PAGE, and the gels were prepared for fluorography.

Heterodimerization of c-Myc with Max protein in vitro. CMfl, CMΔ5′, and Max mRNAs were transcribed and translated separately in vitro in the presence of [^{35}S]methionine at 30°C as described above and then treated with RNase A (final concentration, 0.2 mg/ml) for a further 10 min at 30°C. The lysates were adjusted for incorporation of radiolabel into protein, and comparable amounts of either CMfl- or CMΔ5′-programmed lysate were mixed with equal amounts (10 μl) of Max-programmed lysate. The mixtures were incubated at 37°C for 30 min to allow dimerization to occur and then diluted in 0.5 ml of cold nondenaturing buffer (DPBS [pH 7.3] containing 1% BSA, 1% Triton X-100, and 1% aprotinin). The samples were precleared by preincubation with 40 μl of a 50% slurry of protein A–Sepharose CL-4B (Sigma) on ice for 30 min followed by centrifugation at 500 $\times$ *g*. The supernatants were transferred to a fresh tube, and anti-Mycfl or anti-Max antiserum was added before incubation on ice for 2 h. The immune complexes were precipitated by incubation with protein A-Sepharose for an additional 30 min on ice followed by three washes with cold nondenaturing buffer. Immunoprecipitated Myc and Max proteins were released from the Sepharose pellets by incubation at 95°C with sample buffer and were separated by SDS–12% PAGE.

RESULTS

Avian bursal lymphoma cells express aberrant patterns of c-Myc proteins. Previously, we investigated the synthesis of c-Myc proteins in human Burkitt's lymphomas having chromosomal translocations of the c-*myc* gene (21, 22). In this study, we have compared the c-Myc protein synthesis patterns of a number of avian bursal lymphoma cell lines harboring retroviral insertions and mutations of the c-*myc* locus with the c-Myc protein synthesis patterns of another hematopoietic cell line (MSB-1) having an intact c-*myc* locus. As shown in Fig. 1A, the c-Myc 1 and 2 proteins immunoprecipitated from the MSB-1 cell lysates migrated to apparent molecular masses of 62 and 59 kDa, respectively, on SDS-PAGE. However, in a manner reminiscent of observations made with Burkitt's lymphomas and other neoplastic cells, we observed that most of the bursal lymphoma cell lines did not express the typical pattern of c-Myc proteins as seen with MSB-1 cells having no alterations of the c-*myc* locus. Some of the tumor lines express c-Myc proteins with aberrant sizes (S13 cells express a 61-kDa form, and H1 cells express a 63-kDa form), whereas others did not express c-Myc 1 protein (Bk25, 243L1, and 249L4 cells) or the 60-kDa c-Myc protein of intermediate size (293Sc cells). In fact, we found that only Bk3A cells appear to express a pattern of c-Myc proteins similar to that of MSB-1 cells, albeit at higher overall levels, as compared with the other bursal lymphoma lines.

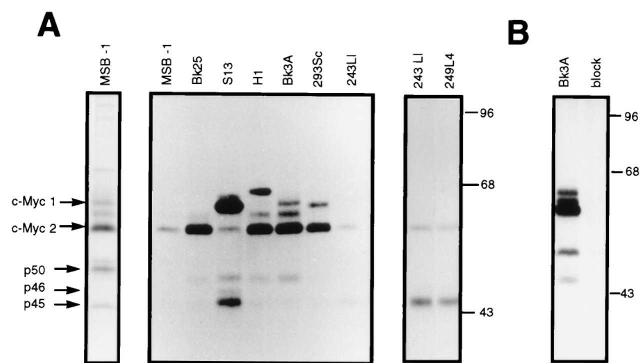

FIG. 1. Avian bursal lymphoma cell lines express aberrant patterns of c-Myc proteins. (A) The Marek's disease virus-infected avian T-cell line MSB-1 and the bursal lymphoma cell lines Bk3A, Bk25, S13, H1, 293Sc, 243L1, and 249L4 were metabolically labeled with [^{35}S]methionine for 20 min, and the c-Myc proteins were immunoprecipitated from the cellular lysates with anti-av-Myc12C after equalization for incorporated label into protein as described in Materials and Methods. The MSB-1, 243L1, and 249L4 immunoprecipitations were repeated with higher amounts of cellular lysates to enhance visualization of the various c-Myc proteins. (B) Specificity of anti-av-Myc12C for c-Myc proteins was demonstrated by immunoprecipitations from Bk3A cellular lysates after preincubation of the antibody with (block) or without the peptide antigen. Standard molecular mass markers (in kilodaltons) are described in Materials and Methods.

In a number of these avian hematopoietic cell lines, we also detected various levels of several smaller c-Myc-related proteins as well (Fig. 1A). The smaller proteins migrated to apparent molecular masses of 45, 46, and 50 kDa on SDS-PAGE gels. Two of the cell lines even expressed the 45-kDa protein at levels equal to or greater than the levels of full-length c-Myc (243L1 and 249L4 cells). Since these smaller proteins were immunoprecipitated with our affinity-purified species-specific antibody to the last 12 C-terminal amino acids in the avian c-Myc sequence (anti-av-Myc12C), these proteins most likely contained the C terminus of the c-Myc protein. The specificity of the antibody for these proteins was demonstrated by the block of recognition by preincubation with the cognate antigen (Fig. 1B). Furthermore, since we detected smaller proteins in several different cell lines, including one having an intact c-*myc* locus derived from a different hematopoietic lineage (MSB-1), it is unlikely that these proteins arise from some spurious mutations within the c-*myc* gene or represent some cell-type-specific cross-reacting protein.

Mammalian and avian cells express N-terminally truncated c-Myc S proteins. Then, we examined the expression of c-Myc proteins in several mammalian cell lines derived from various tissues with the different species-specific C-terminal peptide antisera to characterize further these smaller proteins. To demonstrate antibody specificity, the various antibodies were preincubated with their cognate antigens (block). Figure 2A shows that smaller proteins in addition to full-length c-Myc 1 (68-kDa) and 2 (65-kDa) proteins were specifically immunoprecipitated from MEL cells with our murine-specific anti-mu-Myc12C antibody. MEL cells expressed several smaller proteins having molecular masses of 47, 48, and 50 kDa. Various amounts of small proteins of similar sizes were also seen in immunoprecipitations from NIH 3T3 fibroblasts, AKR-2B fibroblasts, and M1 murine myeloid leukemia cells (data not shown). Our human-specific hu-Myc12C antibody also specifically recognized smaller proteins of approximately 46 and 48 kDa, in addition to the full-length c-Myc 1 (67-kDa) and 2 (64-kDa) proteins, in immunoprecipitations from the human colon carcinoma cell line COLO-320 (Fig. 2A). Taken together, these results indicate that mammalian and avian cells

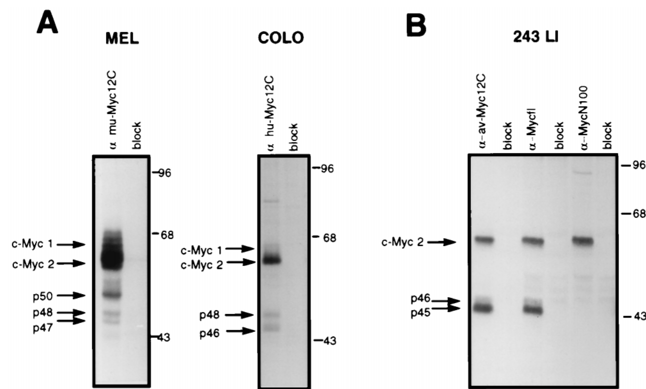

FIG. 2. Mammalian and avian cells express N-terminally truncated c-Myc S proteins. (A) Comparison of c-Myc protein expression patterns from MEL and COLO-320 cells. The c-Myc proteins were immunoprecipitated from [^{35}S]methionine-labeled cellular lysates by using the anti (α)-mu-Myc12C and anti (α)-hu-Myc12C antibodies preincubated with (block) or without their cognate peptide antigens, respectively. (B) Specificities of the anti (α)-av-Myc12C, anti (α)-Mycfl, and anti (α)-MycN100 antibodies for avian c-Myc proteins. The c-Myc proteins were immunoprecipitated from [^{35}S]methionine- and [^{35}S]cysteine-labeled 243L1 cellular lysates by using the various antibodies preincubated with (block) or without the cognate antigens as described in Materials and Methods.

express conserved, smaller c-Myc proteins having intact C termini. In addition, these results demonstrate that the expression of these smaller c-Myc proteins is not restricted to a particular cell type.

Since the smaller c-Myc proteins were specifically immunoprecipitated with the affinity-purified antibodies to the species-specific 12 C-terminal amino acids of c-Myc, this suggests that these proteins were truncated at the N terminus. Thus, they should not be immunoprecipitated with antibodies specific for the N terminus of full-length c-Myc 2. Therefore, we generated an antiserum against the bacterially expressed N-terminal 100 amino acids of murine c-Myc 2 (anti-MycN100). We also compared the specificity of our C-terminal Myc antibody to that of another antiserum generated against bacterially expressed full-length murine c-Myc 2 protein (anti-Mycfl). To demonstrate again antibody specificity, duplicate samples were subjected to immunoprecipitation with the various antibodies preincubated with their cognate antigens (block). As shown in Fig. 2B, the 59-kDa full-length c-Myc 2 protein from 243L1 cells was equally recognized by the three different antibodies. However, the 45- and 46-kDa c-Myc-related proteins were immunoprecipitated only with anti-av-Myc12C and anti-Mycfl and not with anti-MycN100. A similar result was obtained with BK3A cells, except that in addition to the 45- and 46-kDa proteins, the 50-kDa protein was specifically immunoprecipitated only with anti-av-Myc12C and anti-Mycfl antibodies but not with anti-MycN100 (data not shown). In addition, the smaller c-Myc proteins found in human and murine cells were specifically immunoprecipitated only with the various antisera generated against full-length c-Myc proteins or against the C terminus and not with anti-MycN100 (data not shown). Since various species of smaller c-Myc-related proteins were specifically immunoprecipitated by at least two distinct antibodies generated by different methods, it is unlikely that they represent nonspecific or cross-reacting proteins having antigenic similarity. In strong support of this conclusion, two-dimensional proteolytic-peptide maps of the avian 45- and 46-kDa and the full-length 59-kDa c-Myc proteins showed a high degree of similarity, essentially verifying their overlapping structural identity (data not shown). Taken together, these results indicate that these smaller C terminus-containing c-Myc proteins lack epitopes associated with the first 100 amino acids of the N terminus of full-length c-Myc 2 protein; thus, they were termed c-Myc short (c-Myc S) proteins.

c-Myc S proteins initiate at downstream AUG codons. Amino-terminally truncated c-Myc S proteins may arise through two possible mechanisms. Since c-Myc proteins are known to be rapidly degraded (19, 21, 45), c-Myc S proteins could represent specific proteolytic fragments of the full-length proteins. Alternatively, these c-Myc S proteins could arise from downstream translation initiation. In support of the second possibility, there are two conserved potential AUG initiator codons found within exon 2 of the c-*myc* gene residing in excellent context (8, 28). The relative positions of these codons within the coding frame and their overall sequence context have been conserved throughout evolution from *Xenopus* to humans (27). The c-Myc proteins initiated at these potential initiator codons would be predicted to have molecular sizes similar to those of the cellular p45 and p46 c-Myc S proteins on SDS-PAGE gels and would lack all of the N-terminal regions recognized by the anti-MycN100 antiserum.

To test whether initiation of translation at codons downstream of the c-Myc 2 start site could account for the expression of c-Myc S proteins, we compared the c-Myc proteins synthesized by avian cells with the c-Myc proteins synthesized in vitro from a full-length chicken c-*myc* (CMfl) RNA and a 5′-truncated c-*myc* (CMΔ5′) RNA lacking the upstream initiation codons for both full-length c-Myc proteins (Fig. 3). Since there is the possibility of inappropriate initiation events associated with in vitro translations (29), the CMfl and CMΔ5′ cDNAs were also inserted into a eukaryotic expression vector to express these proteins in COS cells. The exogenous c-Myc proteins were immunoprecipitated from the COS cells with anti-av-Myc12C which does not recognize the endogenous COS cell c-Myc proteins (22). To compare directly molecular sizes, c-Myc proteins were also immunoprecipitated from Bk3A and 243L1 cell lysates. As shown in Fig. 3, the endogenous 45- and 46-kDa c-Myc S proteins found in avian cells migrated to the same position as the major downstream-initiated c-Myc proteins translated from the 5′-truncated avian c-*myc* RNAs expressed in vitro and in vivo. On occasion, a third protein was also detected in our translations in vitro and in vivo which migrated to the position of the 50-kDa protein found in Bk3A cells. Similar results were obtained for murine c-*myc*, in which the upstream initiator codons were deleted by digestion with *Eco*RV of the murine c-*myc* cDNA (data not shown). Since c-Myc proteins translated from the 5′-truncated c-*myc* RNA in vitro and in vivo were of the same sizes as those found in cells, these data suggest that the cellular N-terminally truncated c-Myc S proteins do not arise through degradation of full-length c-Myc proteins but rather arise through initiation of translation at downstream in-frame codons. These results also indicate that the full-length and c-Myc S proteins can arise from a single message containing the upstream as well as downstream initiator codons. As a final test, the two closely spaced downstream AUG codons in murine c-*myc* were each altered by site-specific mutagenesis, and the results confirmed that p45 and p46 initiated at these two downstream codons (data not shown).

c-Myc S proteins arise from a leaky scanning mechanism. Mutational analysis suggests that the c-Myc S proteins initiate at the conserved downstream AUG codons. One possible explanation for this downstream initiation is that they arise from a truncated mRNA generated from differential splicing. Even though truncated mRNAs have never been reported, small amounts of a truncated message may be translated more effi-

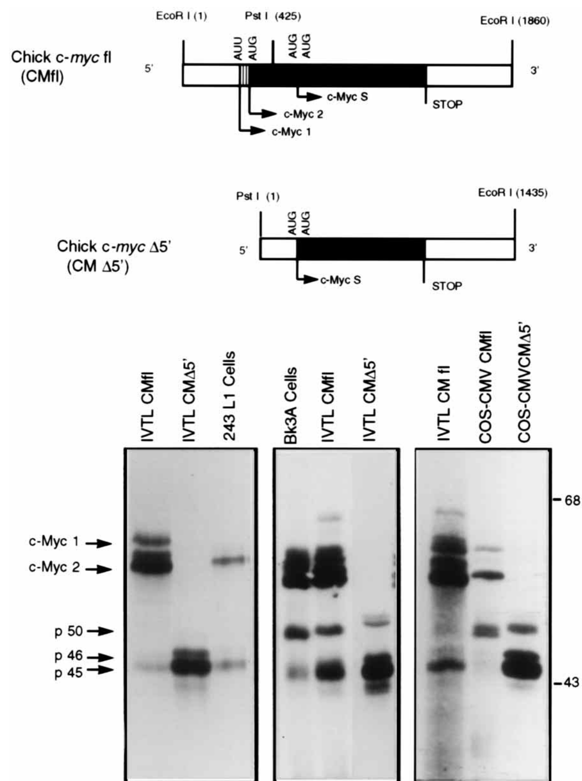

FIG. 3. c-Myc S proteins arise from initiation downstream of the c-Myc 2 start codon in vitro and in vivo. The avian c-*myc* cDNA was truncated by removing the 5′ *Eco*RI-*Pst*I fragment to delete the initiation codons for c-Myc 1 and 2. The full-length (CMfl) and 5′-deleted (CMΔ5′) cDNAs were transcribed and translated in vitro or were subcloned into the eukaryotic expression vector pRc/CMV, creating the plasmids CMV-CMfl and CMV-CMΔ5′, respectively. The molecular sizes of c-Myc proteins immunoprecipitated from avian 243L1 and Bk3A cells were compared on SDS-PAGE gels to the molecular sizes of the in vitro translation products (IVTL) and exogenous avian c-Myc proteins immunoprecipitated from COS cells transfected with the CMV-CMfl and CMV-CMΔ5′ plasmids. The in vitro translations, transfections, and immunoprecipitations with anti-av-Myc12C were performed as described in Materials and Methods.

ciently than a full-length c-*myc* mRNA. Another possibility is that the preinitiation scanning complex bypasses or “leaks” past the first AUG and initiates at a downstream codon. This leaky scanning mechanism often occurs when the first AUG codon is not in a suboptimal context for recognition by the scanning complex (31). Interestingly, in support of this mechanism, the c-Myc 2 initiator AUG lies in a suboptimal consensus sequence in all species of c-*myc* examined.

To test directly whether the c-Myc S proteins arise from a leaky scanning mechanism, we optimized the start codon-context for both murine c-Myc 1 and 2 by site-directed mutagenesis as shown in Fig. 4A. The codon-optimized cDNAs were then placed in cytomegalovirus (CMV) expression vectors and transfected into COS cells. As shown in Fig. 4B, optimization of the normally inefficient CUG start codon for c-Myc 1 resulted in a greatly reduced synthesis of c-Myc S compared with the c-Myc 2 expression vector. Similarly, when the context surrounding c-Myc 2 was optimized, the synthesis of the c-Myc S proteins was reduced compared with the expression from the wild-type c-Myc 2 expression vector (Fig. 4C). These results strongly suggest that the c-Myc S proteins arise by a leaky scanning mechanism.

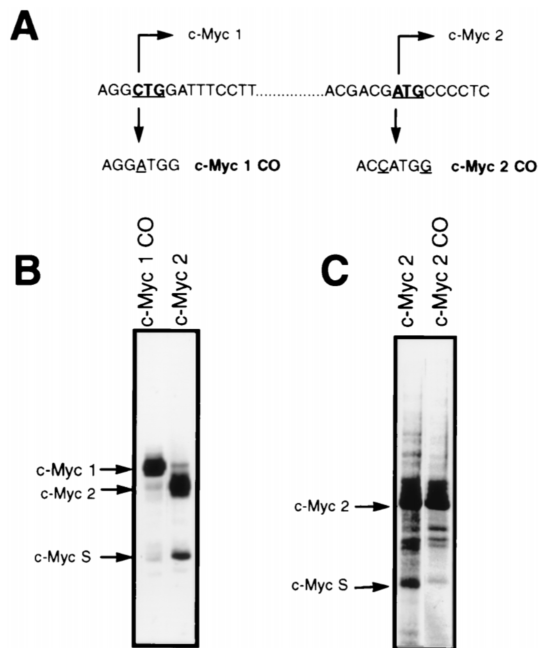

FIG. 4. Optimization of the initiator codons for c-Myc 1 and 2 results in decreased synthesis of c-Myc S. (A) The murine c-*myc* cDNA was altered by site-specific mutagenesis as indicated and placed in the pRc/CMV vector as described in Materials and Methods. Both the murine c-Myc 1 (MM1) and c-Myc 2 (MM2) start codons were optimized (CO) to compare the relative levels of the c-Myc S proteins with that of unaltered c-Myc 2. (B) The CMV-MM1-CO (c-Myc 1 CO) and CMV-MM2 (c-Myc 2) vectors were transiently transfected into COS cells, and the c-Myc proteins were immunoprecipitated with anti-av-Myc12C from [^{35}S]methionine-labeled cells as described in Materials and Methods. Lysates were equalized for TCA-precipitable counts. (C) The CMV-MM2 (c-Myc 2) and CMV-MM2-CO (c-Myc 2 CO) vectors were transfected into COS cells, and the c-Myc proteins were immunoprecipitated as described for panel B.

c-Myc S is transiently expressed during rapid cell growth and is constitutively synthesized at high levels in some tumor cells having rearranged c-*myc* genes. To understand the role that c-Myc S protein expression may play in cell growth control and tumorigenesis and to determine if the leaky scanning mechanism is a regulated event, we examined the synthesis of c-Myc S proteins during growth of several cell types. The mouse NIH 3T3 fibroblast, baby hamster kidney (BHK), and MEL cell lines were examined. All have intact c-*myc* loci and express a typical full-length c-Myc protein synthesis pattern. In addition, we also examined the avian bursal lymphoma cell line 243L1, which does not synthesize c-Myc 1 due to a provirus insertion but does synthesize high levels of c-Myc S proteins.

As shown in Fig. 5A, an increase in the synthesis of the c-Myc S proteins was associated with the rapid growth phase of murine NIH 3T3 fibroblasts. High-density nonproliferating NIH 3T3 cells were replated at a low density in fresh medium and allowed to grow to confluency. The expression of c-Myc protein was examined during the course of their growth by immunoprecipitation analysis with anti-mu-Myc12C antibody. The nonproliferating cells expressed low levels of the c-Myc proteins (Fig. 5A [day 0]). Upon stimulation of growth, there was an increase in the levels of both full-length and c-Myc S protein synthesis after 1 to 2 days. However, as these cells approached confluency, the expression of c-Myc S protein decreased significantly, while expression of the full-length c-Myc proteins was maintained at high levels. Interestingly, by the 3rd day there was a shift from the p50 form of c-Myc S to the p45 form. Similar results were obtained with murine AKR-2B fi-

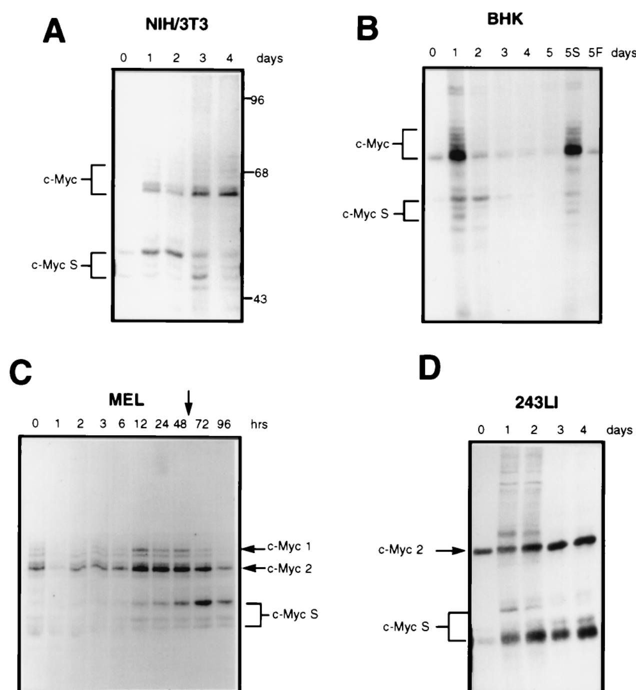

FIG. 5. c-Myc S proteins are expressed transiently during cell growth. (A) Confluent NIH 3T3 cells were replated in replicate at a lower density (1:4) in DMEM containing 2% CS and were allowed to regrow to confluence. Replicate plates were labeled for 20 min with [^{35}S]methionine and [^{35}S]cysteine before replating (day 0) or each day afterward (days 1 to 4), and the c-Myc proteins were immunoprecipitated from the cellular lysates after equalization for TCA-precipitable counts with anti-mu-Myc12C antibody as described in Materials and Methods. (B) Confluent BHK cells were replated at a lower density in DMEM containing 10% CS. Replicate plates were labeled before replating (day 0) or each day afterward (days 1 to 5), and the c-Myc proteins were immunoprecipitated as described for panel A. On day 4, replicate plates were either left untreated (day 5), replated at a lower density in fresh medium (day 5S), or refed with fresh medium without replating (day 5F). (C) High-density, growth-inhibited MEL cells were stimulated to grow by resuspension at a lower density in fresh growth medium. Cells (10^7) were labeled for 20 min before dilution (hour 0) or afterward at the hours indicated, and the c-Myc proteins were immunoprecipitated with anti-mu-Myc12C as described for panel A. Cells were resuspended in fresh medium at a lower density after 48 h, as indicated by the arrow. (D) High-density (7.4×10^6), growth-inhibited 243L1 cells were stimulated to grow by resuspension at lower density (0.2×10^6 cells per ml) in fresh SCM containing 10% CS. Cells were labeled at the indicated days, and the c-Myc proteins were immunoprecipitated with anti-av-Myc12C as described for panel A.

broblasts (data not shown). As shown in Fig. 5B, the synthesis of c-Myc S proteins was regulated in a manner similar to that in NIH 3T3 cells. When high-density cells (day 0) were replated at a lower density in fresh medium, there was an increase in both full-length and c-Myc S proteins after 1 to 2 days, although the full-length c-Myc proteins were expressed at much higher levels compared with those of c-Myc S on day 1. However, while the synthesis of the full-length c-Myc proteins decreased greatly by day 2, c-Myc S remained at nearly the same levels, resulting in greater levels of c-Myc S at that time. As the cells grew to higher density, the synthesis of all of the c-Myc proteins decreased. When the cells were restimulated to grow by replating at a lower density into fresh medium, synthesis of both full-length and c-Myc S proteins increased (day 5S). However, while restimulation of high-density cells with fresh medium without replating did moderately enhance c-Myc 2, it did not result in an increase in c-Myc S protein (day 5F).

Earlier times after restimulation of growth were examined with the nonadherent MEL cells. Cells at high density were stimulated to grow by dilution in fresh growth medium, and the synthesis of the c-Myc proteins during the first 48 h of growth was monitored. As shown in Fig. 5C, c-Myc S protein synthesis increased by 12 h and by 48 h had reached levels comparable to those of c-Myc 2 synthesis. After dilution in fresh medium again at 48 h, the levels of the c-Myc S proteins increased at 72 h, while the levels of c-Myc 1 decreased. We have previously shown that the synthesis of c-Myc 1 decreased when cells were refed with fresh medium (23). Similar results were obtained with the avian bursal lymphoma BK3A cell line and the avian T-cell line MSB-1. These data suggest that the upstream and downstream initiation of both full-length and c-Myc S proteins can be reciprocally regulated. These data also suggest that the transient expression of c-Myc S is most often associated with rapid cell growth in several avian and mammalian cell types.

We then examined the synthesis of c-Myc proteins during the growth of the 243L1 cell line, which has an altered c-*myc* locus and expresses high levels of the c-Myc S proteins. These cells do not express c-Myc 1, but do express the p60 protein of intermediate size. As shown in Fig. 5D, cells at high density (day 0) expressed moderate levels of full-length c-Myc 2 and low levels of c-Myc S. After restimulation of growth (day 1), the levels of full-length c-Myc proteins slightly increased, while the synthesis of c-Myc S proteins dramatically increased to levels equal to or greater than that of c-Myc 2. As the cells grew to high density, the levels of the c-Myc S proteins did not significantly decrease as observed for the other cell lines examined (days 3 to 4).

c-Myc S proteins are nuclear, short-lived phosphoproteins which can heterodimerize with Max. The predicted primary and secondary structures of c-Myc S proteins suggest that they should have a biochemical character that is generally similar to that of full-length c-Myc. Through the use of deletion analyses, it has been determined that the nuclear localization signals of c-Myc reside within the C-terminal region of c-Myc (11, 50). In addition, we and others have examined the nuclear localization of full-length c-Myc proteins by subcellular fractionation using different buffers having various detergent and salt strengths (13, 15). By these procedures, it was determined that the full-length c-Myc proteins belong to a unique subset of nucleus-localized proteins that are not easily extracted from the nuclei. To determine whether the c-Myc S proteins have similar properties, we used two different extraction buffers having different pH and salt strengths. As shown in Fig. 6A, when 243L1 cells were solubilized with TNT buffer (pH 7.4; 50 mM NaCl), the full-length c-Myc 2 protein and the majority of the c-Myc S protein were retained within the insoluble nuclear fraction. When the cells were solubilized with TTN buffer (pH 8.0; 120 mM NaCl), approximately one-half of the full-length c-Myc proteins was extracted from the nuclei into the soluble fraction, whereas the majority of the c-Myc S proteins were extracted. These data suggest that the c-Myc S proteins are nuclear but are more easily extracted than the full-length proteins.

Since phosphorylation sites have been defined within both the N- and C-terminal regions of the full-length c-Myc proteins (37, 38), we examined whether the N-terminally truncated c-Myc proteins were also phosphorylated. The 243L1 cells were metabolically labeled with [^{32}P]orthophosphate, and the c-Myc proteins were immunoprecipitated with anti-av-Myc12C or anti-MycN100. The specificities of the antibodies were tested again by preincubating the antibodies with their cognate antigens prior to addition to the cell lysates. As shown in Fig. 6B, the c-Myc S proteins do indeed contain phosphate and are not recognized by the anti-MycN100 antiserum, again confirming that these proteins lack the N-terminal 100 amino acids.

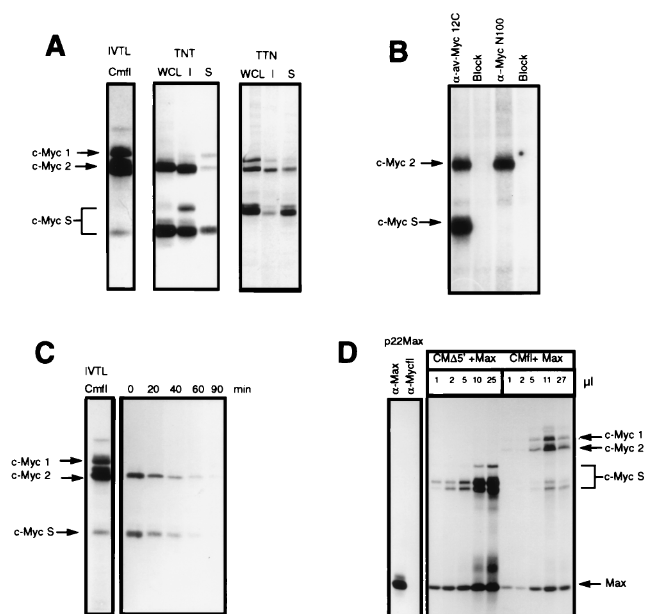

FIG. 6. c-Myc S proteins have biochemical properties similar to those of the full-length c-Myc proteins. (A) Cellular fractionation analysis of c-Myc proteins. Avian 243L1 cells were metabolically labeled for 30 min, and a third of the sample was lysed in complete Ab buffer (WCL) as described in Materials and Methods. The rest of the cells were resuspended in either TNT or TTN, and the insoluble components (I) were separated from the soluble components (S) by centrifugation as described in Materials and Methods. The c-Myc proteins were immunoprecipitated with the anti-av-Myc12C antibody as described in Materials and Methods and were compared in size to in vitro-translated avian c-Myc proteins (IVTL CMfl) on SDS-PAGE gels. (B) Phospholabeling of the c-Myc proteins. Avian 243L1 cells were metabolically labeled with [^{32}S]orthophosphate for 1 h in phosphate-free medium. The cells were lysed in Ab buffer, and the c-Myc proteins were immunoprecipitated with the anti (α)-av-Myc12C and anti (α)-MycN100 antibodies preincubated with (block) or without their cognate antigens. (C) Pulse-chase analysis of c-Myc protein turnover in avian 243L1 cells. Cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 10 min (pulse) and samples were collected immediately (time zero) or at the indicated minutes (chase) as described in Materials and Methods. The c-Myc proteins were immunoprecipitated from the cellular lysates after equalizing for incorporation of radioactive label with anti-av-Myc12C as described in Materials and Methods. (D) Heterodimerization of c-Myc with Max protein in vitro. In vitro-transcribed, 5′-capped CMfl (full-length c-Myc), CMΔ5′ (c-Myc S), and Max mRNAs were translated separately, and increasing amounts of either CMfl or CMΔ5′ lysates were mixed with 10 μl of Max lysate and allowed to heterodimerize as described in Materials and Methods. The complexes were immunoprecipitated with anti-Mycfl in nondenaturing buffer as described in Materials and Methods. Samples of Max lysate (10 μl) were also subjected to immunoprecipitation under the same conditions with either anti-Max (α-Max) or anti-Mycfl (α-Mycfl) as controls (left panel).

Full-length c-Myc proteins have been shown to have very short half-lives (21, 45); however, the protein destabilization region has not been identified. Thus, it was not known whether the c-Myc S proteins have different turnover rates. Therefore, we compared the stabilities of full-length and c-Myc S proteins by pulse-chase analysis as described in Materials and Methods. As shown in Fig. 6C, there were no appreciable differences in stability between the full-length and c-Myc S proteins in 243L1 cells. A similar result was also obtained with avian Bk3A cells and MEL cells in pulse-chase analyses (data not shown). These results indicate that the c-Myc S proteins, like full-length c-Myc, are highly unstable and thus that both would most likely accumulate in cells at similar rates.

Since the c-Myc S proteins also contain the C-terminal HLH-LZ protein dimerization motif, they should be capable of forming complexes with the Max protein (7, 43). Therefore, we examined whether the c-Myc S and full-length c-Myc proteins could both complex with Max protein in an in vitro binding assay (Fig. 6D). Full-length c-Myc, c-Myc S, and Max proteins were translated separately, and increasing amounts of the two different c-Myc protein translation lysates were mixed with equal amounts of Max translation lysate. The mixtures were incubated at 37°C for 30 min to allow heterodimerization to occur. The c-Myc protein complexes were then immunoprecipitated with the anti-Mycfl antiserum in a nondenaturing buffer. As shown in the left panel of Fig. 6D, the specificity of the Myc antibody was demonstrated by the absence of Max protein in immunoprecipitations of Max lysate alone with anti-Mycfl antiserum (anti-Mycfl). A duplicate sample of Max lysate alone was also immunoprecipitated with our polyclonal anti-Max antiserum to demonstrate the maximal amount of Max protein which could be immunoprecipitated from these reactions (anti-Max). The right panel of Fig. 6D shows that both full-length c-Myc and c-Myc S protein were capable of coprecipitating Max protein in a dose-dependent manner. As predicted, these data indicate that the c-Myc S proteins heterodimerize with Max protein in vitro.

c-Myc S proteins inhibit transactivation by full-length c-Myc protein. Transactivation of target sequences by full-length c-Myc proteins has been shown to depend on the integrity of the C-terminal DNA binding and protein heterodimerization domain (bHLH-LZ), as well as the N-terminal transactivation domain (1, 26, 32). Our results showing that the c-Myc S proteins, like the full-length proteins, are localized to the nucleus and heterodimerize with Max protein suggest that they both have the potential to compete for free Max protein in vivo. These c-Myc S–Max protein complexes would then be expected to compete with full-length c-Myc–Max complexes for binding to transcriptional control elements. However, as illustrated in Fig. 7A, the c-Myc S proteins have a severely truncated transactivation domain and would most likely have little or no transcriptional activity alone. Thus, like Max homodimers, the formation of inactive c-Myc S–Max complexes and their subsequent binding to transcriptional regulatory elements would be expected to negatively regulate transactivation by full-length c-Myc–Max complexes. To test this hypothesis, we examined the effects of c-Myc S expression alone or of the coexpression of c-Myc S and full-length c-Myc proteins together on the transcriptional activities of two different target sequences.

The $[EMS]_4$-chloramphenicol acetyltransferase (CAT) reporter, consisting of four reiterated copies of the CACGTG c-Myc binding sequence upstream of a minimal TATA-box promoter element (32), was transiently transfected into COS cells alone or together with either the c-Myc 1 protein expression vector (LXSN-HM1), the c-Myc 2 protein expression vector (LXSN-HM2), or the c-Myc S protein expression vector (CMV-CMΔ5′). Immunoprecipitation analysis from duplicate plates of transfected cells confirmed that the c-Myc and Max proteins were expressed at comparable levels (data not shown). As shown in Fig. 7B, expression of the c-Myc S protein alone had no effect on the basal transcription level of the $[EMS]_4$-CAT reporter. As expected, expression of the full-length c-Myc proteins stimulated $[EMS]_4$-CAT transcription by fivefold. Figure 7C demonstrates that cotransfection of increasing amounts of c-Myc S with full-length c-Myc 2 inhibited the transactivation of $[EMS]_4$-CAT to basal levels, indicating that c-Myc S behaves as a dominant-negative inhibitor of transactivation by the c-Myc 2 protein.

We then examined the response of the $[EFII]_6$-CAT reporter plasmid containing the EFII enhancer sequence reiterated six times upstream of a minimal TATA box. Unlike with

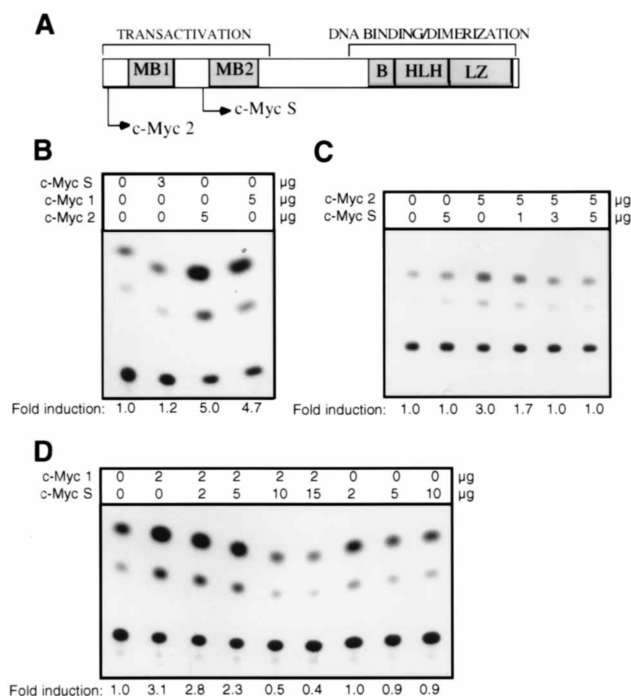

FIG. 7. c-Myc S proteins behave as dominant-negative inhibitors of transactivation by full-length c-Myc proteins. (A) COS cells were transfected with 5 μl of the $[EMS]_4$-CAT reporter, 1 μl of simian virus 40–β-galactosidase, and the indicated amounts of either c-Myc 2 protein expression vector (LXSN-HM2), c-Myc 1 protein expression vector (LXSN-HM1), or the c-Myc S expression vector (CMV-CMΔ5′). The amount of c-Myc S expression vector transfected was decreased to allow for comparable synthesis levels of the different c-Myc proteins. The cells were harvested 48 h after transfection and were assayed for β-galactosidase and CAT activities. Transfection efficiencies were normalized against β-galactosidase activities, and the CAT assays were performed as described in Materials and Methods. Fold induction is based on the conversion value obtained for the $[EMS]_4$-CAT transfection alone (control percent conversion, 1.0 U). (B) COS cells were transfected with 5 μl of the $[EMS]_4$-CAT reporter, 1 μl of simian virus 40–β-galactosidase, and the indicated amounts of either c-Myc 2 protein expression vector (LXSN-HM2) or the c-Myc S expression vector (CMV-CMΔ5′) or combinations thereof. The cells were harvested and CAT assays were performed as described for panel A. Fold induction is based on the conversion value obtained for the $[EMS]_4$-CAT transfection alone (control percent conversion, 1.0 U). (C) COS cells were transfected by the calcium phosphate precipitation method with 2 μg of $[EFII]_6$-CAT construct and 1 μg of simian virus 40–β-galactosidase reporter alone or together with the indicated amounts of the c-Myc 1 protein expression vector (CMV-MM1) or with the c-Myc S protein expression vector (CMV-MMΔ5′) or combinations thereof. Cells were harvested, and the CAT assays were performed as described for panel A. Fold induction is based on the conversion value obtained for the $[EFII]_6$-CAT transfection alone (control percent conversion, 1.0 U).

the EMS sequence, we have shown that the c-Myc 1 protein, but not the c-Myc 2 protein, can stimulate transcription through the C/EBP site of this enhancer element (21). COS cells were transiently transfected with $[EFII]_6$-CAT reporter plasmid alone or together with either the full-length c-Myc 1 expression vector (CMV-MM1), the c-Myc S expression vector (CMV-MMΔ5′), or combinations thereof (Fig. 7D). As expected, transfection with small amounts of c-Myc 1 protein expression vector alone modestly stimulated $[EFII]_6$-CAT expression (a threefold increase over control), while transfection with c-Myc S alone was ineffectual or slightly repressed $[EFII]_6$-CAT expression. Furthermore, the stimulation of $[EFII]_6$-CAT expression by c-Myc 1 was modestly inhibited at low doses of c-Myc S and completely repressed by high doses of c-Myc S. In fact, repression below basal levels was observed with the higher amounts of c-Myc S. Thus, these results confirm that c-Myc S proteins cannot transactivate and that coexpression of c-Myc S proteins can negatively regulate transcriptional activation by full-length c-Myc proteins through EFII and EMS elements in COS cells.

DISCUSSION

In addition to the two major full-length forms of c-Myc protein, we and other investigators have previously reported the detection of smaller c-Myc-related proteins in immunoprecipitations from $[^{35}S]$methionine-labeled cellular lysates or in Western blot analyses (21, 22, 35, 40, 49). In this report, we demonstrate that these smaller proteins in mammalian and avian cells are alternatively initiated forms of c-Myc, termed c-Myc S proteins. Deletional analysis and site-specific mutagenesis demonstrated that these proteins arise from translational initiation from two closely spaced AUG-initiator codons downstream of the start sites for c-Myc 1 and 2 proteins. These downstream-initiated c-Myc S proteins appear to arise through a leaky scanning mechanism. This is possible since the non-AUG initiator codon for c-Myc 1 protein is normally inefficient in growing cells (22, 23), and the context of the AUG initiator for c-Myc 2 protein is suboptimal for initiation in all species examined (28). Therefore, the scanning ribosomal preinitiation complex could read through the upstream initiator codons for c-Myc 1 and 2 and initiate translation at the downstream AUG codons which reside in optimal context. Optimization of the initiation codon for c-Myc 1 or of the context surrounding the c-Myc 2 initiation codon resulted in less synthesis of the c-Myc S proteins, which is consistent with a leaky scanning mechanism.

Interestingly, this leaky scanning mechanism appears to be regulated during proliferation of a variety of cell types. The highest levels of the c-Myc S proteins were transiently observed 24 to 48 h after replating cells at a lower density, corresponding with the rapid growth phase of the cell population. Levels of c-Myc S often were comparable to the levels of the full-length c-Myc proteins in several cell types at these times. As cells approached high density, the synthesis of c-Myc S proteins decreased dramatically. Previously, we demonstrated that the non-AUG-initiated c-Myc 1 protein increases to levels equal to or greater than that of c-Myc 2 as cells approach high-density growth arrest (23). It appears that when cells synthesize high levels of c-Myc 1, there is a corresponding decrease in c-Myc S proteins. Enhanced synthesis of c-Myc 1 by optimization of the CUG codon also resulted in less c-Myc S. Therefore, an increase in the c-Myc 1 start codon usage appears to result in less leaky scanning. However, the transient synthesis of c-Myc S during rapid cell growth when there are no changes in the synthesis of c-Myc 1 suggests that another mechanism(s) also controls the synthesis of the downstream-initiated c-Myc S proteins by regulating the leaky scanning past the c-Myc 2 AUG.

Alterations of the c-*myc* gene can disrupt the normal translational regulation of the c-Myc proteins. Several bursal lymphoma cell lines having alterations of the c-*myc* locus no longer express c-Myc 1, and some express constitutively high levels of c-Myc S proteins. The loss of c-Myc 1 synthesis seen in some of the bursal lymphoma cell lines is most likely the result of provirus insertion in intron 1, yielding a transcript lacking the c-Myc 1 start codon found in exon 1, in agreement with the previous mapping of the provirus integration sites in these cell lines (34). Overexpression of c-Myc S appears to be a consequence of at least one c-*myc* gene alteration. However, loss of c-Myc 1 synthesis does not necessarily lead to constitutive high levels of c-Myc S, since BK25 cells do not synthesize high levels

of c-Myc S even though they have lost c-Myc 1 synthesis. In addition, provirus integration alone cannot explain the synthesis of the aberrant c-Myc proteins found in S13 and H1 cells. These most likely arise from other alterations such as point mutations. The origin of the 60-kDa c-Myc protein, intermediate in size, which is not found in 293Sc cells, is not known, but it may represent a modified form of c-Myc 2 or another upstream non-AUG-initiated protein. Similarly, the origin of the 50-kDa form of the c-Myc protein is unknown; however, it is observed after deletion of the c-Myc 1 and 2 start sites and therefore represents another form of c-Myc S.

The biochemical properties of the c-Myc S proteins are similar to those of the full-length c-Myc proteins. Since the c-Myc S proteins retain the C-terminal domains, they would be expected to be phosphorylated, nuclear proteins capable of heterodimerizing with Max. These properties were indeed similar; however, the c-Myc S proteins appear to be more easily extracted from the nucleus than the full-length proteins. The finding that c-Myc S also has a turnover similar to that of c-Myc 2 suggests that the region necessary for the rapid degradation of c-Myc is not found in the N-terminal 100 amino acids. The N-terminal 100 amino acids do appear to be important for the transactivation properties of c-Myc, as previously shown by several groups (26, 32). Thus, predictably, c-Myc S proteins had no transactivation abilities and behaved as dominant-negative inhibitors of transactivation by full-length c-Myc through EMS and EFII-enhancer elements in transient transfection assays in COS cells. Some repression below basal levels was observed through the C/EBP element of the EFII enhancer.

These data suggest that the expression of c-Myc S represents another mechanism by which the transcriptional activities of the full-length c-Myc proteins may be modulated. The regulation of c-Myc transcriptional activity appears to be dependent on a complex network of positive and negative regulatory proteins. Heterodimerization with Max appears critical for EMS-mediated transactivation by c-Myc. However, in addition to c-Myc, Max proteins can form a ternary complex with a family of related proteins (Mad and Mxi) and transcriptional repressors (mSin 3A and -B) to repress transactivation through EMS (25). An alternatively spliced form of Max, dMax, which lacks the basic DNA binding region of Max, can heterodimerize with c-Myc and also repress transactivation through EMS sequences (3). Thus, similarly to c-Myc S, dMax can behave as a dominant-negative inhibitor of c-Myc function. Transcriptional repression by truncated downstream-initiated translational forms has also been shown for a number of other transcription factors and growth regulatory genes (17, 31). The transcriptional activity of the thyroid hormone itself may be autoregulated by naturally occurring truncated forms of the receptor, most likely arising from leaky scanning past upstream initiation codons and initiation at downstream AUG codons (5). Similarly, the full-length C/EBPβ (LAP) transcription factor is a powerful transactivator, whereas the downstream-initiated form, LIP, has a truncated transactivation domain and antagonizes the activity of LAP (12). The transcriptional activator C/EBPα, which has antimitotic activity associated with adipocyte differentiation, can also be alternatively initiated at a downstream initiator codon, yielding a truncated protein which fails to interfere with adipocyte cell proliferation and to induce complete 3T3-L1 differentiation (42).

Since the c-Myc S proteins can behave as dominant-negative inhibitors of transactivation by the full-length c-Myc proteins, they might be expected to lack biological activity or perhaps even inhibit the biological activity of the full-length proteins. The transient synthesis of the c-Myc S proteins during the rapid growth phase of cells and the occurrence of tumors which express high levels of c-Myc S suggest that these truncated proteins do not inhibit proliferation and may even stimulate proliferation, perhaps through the repression of negative growth regulatory genes. The inhibition of gene expression may be an important component of the mechanism by which many transcription factors and oncogenes, including c-*myc*, promote cell growth and transformation (16, 33, 36). For example, the *erbA* oncogene represents a dominant-negative version of the thyroid hormone receptor and may contribute to the formation of hematopoietic tumors by constitutive suppression of negative growth regulatory genes (10). In addition, v-*rel* may also represent an example of gene repression and cell growth promotion. The v-Rel protein can act as a dominant-negative regulator of gene activation by other members of the *rel* family such as NF-κB (4). Studies have also suggested that the contribution of c-*jun* or v-*jun* expression to cell transformation may not require the ability to activate transcription but rather the ability to repress transcription (24).

The ability to express at least three amino-terminally unique forms of c-Myc protein may be important for the normal function of c-*myc* in cell growth control. These three amino-terminally unique c-Myc proteins appear to have distinct mechanisms by which their synthesis is regulated and appear to have different abilities to transactivate through specific DNA sequences. Further studies are in progress to determine if there are biological similarities between the various translational forms of c-Myc. The properly balanced ratio of their synthesis, which can fluctuate depending on the cellular environment, may provide a sensitive mechanism for the modulation of c-*myc* function during different stages of cell growth, differentiation, or apoptosis. Therefore, an imbalanced expression of the different c-Myc proteins, as was found in a number of tumor cell lines having alterations of the c-*myc* locus, may directly contribute to the loss of cell growth control associated with tumor development.

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REFERENCES

1. **Amati, B., M. W. Brooks, N. Levy, T. Littlewood, G. Evan, and H. Land.** 1993. Oncogenic activity of the c-Myc protein requires dimerization with Max. Cell **72:**233–245.
2. **Amin, C., A. J. Wagner, and N. Hay.** 1993. Sequence-specific transactivation by Myc and repression by Max. Mol. Cell. Biol. **13:**383–390.
3. **Arsura, M., A. Deshpande, S. R. Hann, and G. E. Sonenshein.** 1995. Variant max protein, derived by alternative splicing, associates with c-Myc in vivo and inhibits transactivation. Mol. Cell. Biol. **15:**6702–6709.
4. **Ballard, D. W., W. H. Walker, S. Doerra, P. Sista, J. A. Molitor, E. P. Dixon, N. J. Peffer, M. Hannink, and W. C. Greene.** 1990. The v-rel oncogene encodes a kappa B enhancer binding protein that inhibits NF-kappa B function. Cell **63:**803–814.
5. **Bigler, J., W. Hokanson, and R. N. Eisenman.** 1992. Thyroid hormone receptor transcriptional activity is potentially autoregulated by truncated forms of the receptor. Mol. Cell. Biol. **12:**2406–2417.
6. **Blackwell, T. K., L. Kretzner, E. M. Blackwood, R. N. Eisenman, and H. Weintraub.** 1990. Sequence-specific DNA binding by the c-Myc protein. Science **250:**1149–1151.
7. **Blackwood, E. M., and R. N. Eisenman.** 1991. Max: a helix-loop-helix protein that forms a sequence-specific DNA-binding complex with Myc. Science **251:**1211–1217.
8. **Cavener, D. R.** 1987. Comparison of the consensus sequence flanking translational start sites in Drosophila and vertebrates. Nucleic Acids Res. **15:** 1353–1361.
9. **Cole, M. D.** 1986. The myc oncogene: its role in transformation and differentiation. Annu. Rev. Biochem. **20:**361–384.

10. **Damm, K., C. C. Thompson, and R. M. Evans.** 1989. Protein encoded by v-erbA functions as a thyroid-hormone receptor antagonist. Nature **339:**593–597.
11. **Dang, C. V., and W. F. M. Lee.** 1988. Identification of the human c-*myc* nuclear localization signal. Mol. Cell. Biol. **8:**4048–4054.
12. **Descombes, P., and U. Schibler.** 1991. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell **67:**569–579.
13. **Eisenman, R. N., C. Y. Tachibana, H. D. Abrams, and S. R. Hann.** 1985. v-*myc*- and c-*myc*-encoded proteins are associated with the nuclear matrix. Mol. Cell. Biol. **5:**114–126.
14. **Evan, G., A. H. Wyllie, C. Gilbert, T. Littlewood, H. Land, M. Brooks, C. Waters, L. Penn, and D. Hancock.** 1992. Induction of apoptosis in fibroblasts by c-myc protein. Cell **69:**119–128.
15. **Evan, G. I., and D. C. Hancock.** 1985. Studies on the interaction of the human c-myc protein with cell nuclei: p62 c-myc as a member of a discrete subset of nuclear proteins. Cell **43:**253–261.
16. **Foulkes, N. S., and P. Sassone-Corsi.** 1992. More is better: activators and repressors from the same gene. Cell **68:**411–414.
17. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of the human adenovirus 5 DNA. Virology **52:**456–467.
18. **Gu, W., K. Bhatia, I. T. Magrath, C. V. Dang, and R. Dalla-Favera.** 1994. Binding and suppression of the myc transcriptional activation domain by p107. Science **264:**251–254.
19. **Hann, S. R., H. D. Abrams, L. R. Rohrschneider, and R. N. Eisenman.** 1983. Proteins encoded by v-Myc and c-Myc oncogenes: identification and localization in acute leukemia transformants and bursal lymphoma cell lines. Cell **34:**789–798.
20. **Hann, S. R., M. Dixit, R. C. Sears, and L. Sealy.** 1994. The alternatively initiated c-Myc proteins differentially regulate transcription through a noncanonical DNA-binding site. Genes Dev. **8:**2441–2452.
21. **Hann, S. R., and R. N. Eisenman.** 1984. Proteins encoded by the human c-*myc* oncogene: differential expression in neoplastic cells. Mol. Cell. Biol. **4:**2486–2497.
22. **Hann, S. R., M. W. King, D. L. Bentley, C. W. Anderson, and R. N. Eisenman.** 1988. A non-AUG translational initiation in c-Myc exon 1 generates an N-terminally distinct protein whose synthesis is disrupted in Burkitt's lymphomas. Cell **52:**185–195.
23. **Hann, S. R., K. Sloan-Brown, and G. D. Spotts.** 1992. Translational activation of the non-AUG-initiated c-Myc-1 protein at high cell densities due to methionine deprivation. Genes Dev. **6:**1229–1240.
24. **Havarstein, L. S., I. M. Morgan, W.-Y. Wong, and P. K. Vogt.** 1992. Mutations in the Jun delta region suggest an inverse correlation between transformation and transcriptional activation. Proc. Natl. Acad. Sci. USA **89:**618–622.
25. **Henriksson, M., and B. Luscher.** 1996. Proteins of the myc network: essential regulators of cell growth and differentiation, p. 109–182. *In* G. F. Vande Woude and G. Klein (ed.), Advances in cancer research. Academic Press, New York, N.Y.
26. **Kato, G. J., J. Barret, M. Villa-Garcia, and C. V. Dang.** 1990. An amino-terminal c-Myc domain required for neoplastic transformation activates transcription. Mol. Cell. Biol. **10:**5914–5920.
27. **King, M. W., J. M. Roberts, and R. N. Eisenman.** 1986. Expression of the c-*myc* proto-oncogene during development of *Xenopus laevis*. Mol. Cell. Biol. **6:**4499–4508.
28. **Kozak, M.** 1986. Bifunctional messenger RNAs in eukaryotes. Cell **47:**481–483.
29. **Kozak, M.** 1989. Context effects and inefficient initiation at non-AUG codons in eukaryotic cell-free translation systems. Mol. Cell. Biol. **9:**5073–5080.
30. **Kozak, M.** 1991. An analysis of vertebrate mRNA sequences: intimations of translational control. J. Cell Biol. **115:**887–903.
31. **Kozak, M.** 1992. Regulation of translation in eukaryotic systems. Annu. Rev. Cell. Biol. **8:**197–225.
32. **Kretzner, L., E. M. Blackwood, and R. N. Eisenman.** 1992. Myc and Max proteins possess distinct transactivational capabilities. Nature **359:**426–429.
33. **Lewin, B.** 1991. Oncogenic conversion by regulatory changes in transcription factors. Cell **64:**303–312.
34. **Linial, M., N. Gunderson, and M. Groudine.** 1985. Enhanced transcription of c-myc in bursal lymphoma cells requires continuous protein synthesis. Science **230:**1126–1132.
35. **Luscher, B., and R. N. Eisenman.** 1988. c-*myc* and c-*myb* protein degradation: effect of metabolic inhibitors and heat shock. Mol. Cell. Biol. **8:**2504–2512.
36. **Luscher, B., and R. N. Eisenman.** 1990. New light on myc and myb. Part I. myc. Genes Dev. **4:**2025–2035.
37. **Luscher, B., E. A. Kuenzel, E. G. Krebs, and R. N. Eisenman.** 1989. Myc proteins are phosphorylated by casein kinase II. EMBO J. **8:**1111–1119.
38. **Lutterbach, B. L., and S. R. Hann.** 1994. Hierarchical N-terminal phosphorylation at sites in transformation-sensitive c-Myc protein is regulated by mitogens and in mitosis. Mol. Cell. Biol. **14:**5510–5522.
39. **Maheswaran, S., H. Lee, and G. Sonenshein.** 1994. Intracellular association of the protein product of the c-*myc* oncogene with the TATA-binding protein. Mol. Cell. Biol. **14:**1147–1152.
40. **Morgan, J. H., and J. T. Parsons.** 1986. Characterization of c-myc proteins from avian bursal lymphoma cell lines. Virology **150:**178–186.
41. **Nottenburg, C., and H. E. Varmus.** 1986. Features of the chicken c-*myc* gene that influence the structure of c-*myc* RNA in normal cells and bursal lymphomas. Mol. Cell. Biol. **6:**2800–2806.
42. **Ossipow, V., P. Descombes, and U. Schibler.** 1993. CCAAT/enhancer binding protein mRNA is translated into multiple proteins with different transcription activation potentials. Proc. Natl. Acad. Sci. USA **90:**8219–8223.
43. **Prendergast, G. C., D. Lawe, and E. B. Ziff.** 1991. Association of Myn, the murine homolog of max, with c-Myc stimulates methylation-sensitive DNA binding and ras cotransformation. Cell **65:**395–407.
44. **Prendergast, G. C., and E. B. Ziff.** 1991. Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. Science **251:**186–189.
45. **Ramsay, G., G. I. Evan, and J. M. Bishop.** 1984. The protein encoded by the human proto-oncogene c-myc. Proc. Natl. Acad. Sci. USA **81:**7742–7746.
46. **Ryan, K. M., and G. D. Birnie.** 1996. Myc oncogenes: the enigmatic family. Biochem. J. **314:**713–721.
47. **Skinner, M. K., and M. D. Griswold.** 1983. Fluorographic detection of radioactivity with 2,5-diphenyloxazole in acetic acid and its comparison with existing procedures. Biochem. J. **209:**281–284.
48. **Spencer, C. A., and M. Groudine.** 1991. Control of c-Myc regulation in normal and neoplastic cells. Adv. Cancer Res. **56:**1–48.
49. **Spotts, G. D., and S. R. Hann.** 1990. Enhanced translation and increased turnover of c-*myc* proteins occur during differentiation of murine erythroleukemia cells. Mol. Cell. Biol. **10:**3952–3964.
50. **Stone, J., T. de Lange, G. Ramsay, E. Jakobovits, J. M. Bishop, H. Varmus, and W. Lee.** 1987. Definition of regions in human c-*myc* that are involved in transformation and nuclear localization. Mol. Cell. Biol. **7:**1697–1709.